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(54) Title: EXPRESSION OF DNA SEQUENCES DERIVED FROM NOCARDIOFORM MICROORGANISMS

(57) Abstract

A cloning vector contains a DNA sequence derived from a Nocardioform microorganism, and is capable of expressing the DNA sequence in a host microorganism. Recombinant cholesterol oxidase is produced by a host microbial cell transformed with a cloning vector containing cholesterol oxidase encoding DNA derived from a Nocardioform microorganism. A method for determining cholesterol oxidase in a sample of fluid from a human includes contacting the sample with the Nocardioform microorganism derived recombinant cholesterol oxidase.

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EXPRESSION OF DNA SEQUENCES DERIVED FROM NOCARDIOFORM MICROORGANISMS

Background of the Invention

This invention relates to gene expression.

The organisms grouped as Nocardioforms in Bergey's Manual of Systematic Bacteriology (Williams and Wilkins, 1986, pp. 1458-1481) include nine genera which are close phylogenetically to Corynebacterium, Athrobacter and Mycobacterium. Members of the Nocardioforms including the genera Nocardia and Rhodococcus exhibit a wide range of useful metabolic activities including the assimilation of unusual compounds such as alkanes and aromatic hydrocarbons; the degradation of lignin, detergents and pesticides; the production of enzymes useful in xenobiotic transformations; and the biosynthesis of antibiotics, amino acids and biosurfactants. In particular, this group of bacteria have been exploited for their production of important steroid modifying enzymes, including cholesterol esterase and cholesterol oxidase, which are used in diagnostic assays to determine cholesterol levels in blood and serum.

The capacity to degrade cholesterol is widespread among microorganisms other than the Nocardioforms. For example, cholesterol oxidases have also been isolated from species of Mycobacterium, Pseudomonas, and Streptomyces. It is well known from scientific and patent literature and from commercial practice that the Nocardioform-type cholesterol oxidase is distinct from the other microbial cholesterol oxidases.

The Nocardioform enzyme is very stable and active over a wide pH range (pH 6.0 - 8.0), the Km for cholesterol is 1.4×10^{-5} mol/liter at 25°, and the

enzyme is highly specific for Δ^4- or Δ^5-3ß-sterols. As produced in the Nocardioforms, cholesterol oxidase is membrane associated and requires detergent extraction and lipid removal for purification.

Singer, et al., J. Bacteriol., 1988, Vol. 170, pp. 638-645, describes expression of genes in Rhodococcus sp. from both E. coli and Streptomyces sp. and the development of a shuttle plasmid based on an E. coli plasmid and containing a Rhodococcus plasmid-derived origin of replication. The plasmid is capable of replicating in E. coli and in several but not all of the Rhodococcus species tested.

Summary of the Invention

We have discovered that recombinant DNA sequences derived from Nocardioform microorganisms can be expressed efficiently in a host microorganism.

The invention features, in one aspect, a cloning vector which expresses DNA sequences, derived from a Nocardioform organism, in a host microorganism. In preferred embodiments, the DNA sequence is derived from Rhodococcus sp. NCIB 10554 (National Collection of Industrial Bacteria, Aberdeen, Scotland); the DNA sequence encodes cholesterol oxidase; the organism in which the cloning vector can express the Nocardioform DNA is a gram-positive microorganism, more preferably a species of Streptomyces, and most preferably S. lividans; and the cloning vector is the plasmid pSL81.

Cholesterol oxidase is produced according to the invention by providing a cloning vector containing a DNA sequence encoding cholesterol oxidase, which is derived from a Nocardioform microorganism, transforming a host cell with the cloning vector to obtain a recombinant host cell, culturing the recombinant host cell under conditions permitting expression of the DNA

sequence, and recovering the cholesterol oxidase. In preferred embodiments, the Nocardioform organism is Rhodococcus sp. NCIB 10554; the host cell is a gram-positive microorganism, preferably a species of Streptomyces, and more preferably S. lividans; and the cloning vector is the plasmid pSL81.

Cholesterol oxidase produced according to the invention can be used for determining cholesterol in a sample, by contacting the sample with the recombinant cholesterol oxidase and determining the extent of oxidation of cholesterol; specifically, by measuring oxygen consumption with an oxygen electrode; or by measuring the production of hydrogen peroxide; or by spectrophotometrically determining cholesterol conversion to Δ^4-cholestenone.

The invention provides for the expression of DNA sequences derived from a Nocardioform microorganism in a host microorganism that is easier to manipulate under conditions of commercial production than is the parent organism itself, and for which cloning techniques are known. Recombinant cholesterol oxidase derived from Nocardioform microorganisms is expressed as an extracellular protein, an expression condition which can lead to substantially higher production of enzyme. Moreover, the expression does not require addition of an inducer, and the expressed enzyme is free of the lipids of the Nocardioform membrane.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment thereof, and from the claims.

Description of the Preferred Embodiment

The drawing will first be described.

Drawing

Fig. 1 is a restriction endonuclease map of plasmid pSL81.

Fig. 2 (2.1-2.6) is a sequence description of the Rhodococcus 10554 cholesterol oxidase gene.

Fig. 3 is a restriction endonuclease map of plasmid pSL81, showing the position of the pSL81Δ15 fragment.

Gene Isolation and Expression

The expression of DNA sequences derived from a Nocardioform microorganism can be accomplished, in general, by first constructing a genomic bank or library by incorporating the DNA sequences into a suitable cloning vector containing a suitable origin of replication compatible with the desired host organism. Next, a host cell from any microorganism, advantageously any gram-positive microorganism, such as, for example, species of Streptomyces or Bacillus, is transformed with the vector containing the DNA sequences. Then, transformants are screened for the activity of the desired gene product, and active clones are isolated and characterized.

Construction of Library

The following protocol, presented for illustrative purposes, describes construction of a genomic library of Rhodococcus sp. NCIB 10554 (Rhodococcus 10554) DNA in a host such as S. lividans which is capable of expressing the DNA from the parent microorganism. In the example described below, a population of transformed cells prepared using this library is screened for the expression of the cholesterol oxidase gene of Rhodococcus 10554.

Generally, a genomic library of Rhodococcus 10554 DNA was constructed from high molecular weight DNA which had been isolated and fragmented with the restriction enzyme Sau3A to yield fragments in the size range 4-8 kilobase pairs. Size fractionated DNA from Rhodococcus 10554 was then ligated to the cloning

plasmid pIJ702 which had been digested with BglII. The pIJ702 plasmid is commercially available through the American Type Culture Collection (Rockville, MD, USA). Hybrid plasmids of the library were transformed into S. lividans cells to generate the genomic library of Rhodococcus 10554 DNA in the host microorganism.

Details of the procedure were as follows: A single colony of Rhodococcus 10554 was used to inoculate 50 ml of NBYE medium which contained per liter: Difco nutrient broth, 8g, and Difco yeast extract, 5g. Other appropriate media described for growing Rhodococcus sp. could also be used. The culture was grown for 48 hours at 30°C and the cells collected by centrifugation at 10,000 rpm for 15 minutes in a Sorvall RC-5B centrifuge using an SS-34 rotor. The cells were resuspended in 18 ml of Tris-glucose (10mM Tris-HCl, pH 8.0, 10mM EDTA, 25% w/v glucose), and 2 ml of lysozyme solution (10 mg/ml lysozyme (Sigma) in Tris-glucose solution) was added. This mixture was incubated at 37°C for 60 minutes. Next, 2.4 ml of 10% w/v sodium dodecyl sulfate (Sigma) was added and the mixture stirred vigorously to form a homogenous solution of lysed cells. The lysed cells were incubated at 55°C for 2 hours, and then 2.7 ml of 5M $NaClO_4$ (Sigma) were added. Denatured protein and cell debris were removed by extracting the lysed cell solution twice with 20 ml chloroform:isoamyl alcohol (24:1) and resolving the phases by centrifugation at 12,000 rpm for 10 minutes in a Sorvall SS-34 rotor. The aqueous phase was removed and two volumes of cold ethanol added. The solution was mixed gently, and the high molecular weight DNA was collected using a glass rod as described by Marmur et al., 1961, J. Mol. Biol., Vol. 3, pp. 203-218. The collected DNA was washed twice in 70% ethanol and dissolved in 5 ml TE

(10mM Tris, 1mM EDTA, pH 8.0). DNAse free RNAse (Sigma) was added at 100 mg/ml to the DNA solution and incubated at 42°C for 30 minutes. Next, 0.6 ml of 10% w/v of sodium dodecyl sulfate and 0.6 ml of 5M NaCl were added, and the mixture was extracted once with phenol:chloroform (1:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated with 2 volumes of ethanol and collected as before using a glass rod. The DNA was washed once with 70% ethanol and air dried prior to resuspension in TE to a final concentration of approximately 1 mg/ml.

In order to establish the condition for isolating partially digested high molecular weight Rhodococcus 10554 DNA, test reactions were set up to digest DNA with different concentrations of Sau3A restriction enzyme. A reaction mixture was prepared with 10 µl DNA, 15 µl 10x Sau3A buffer (200mM Tris HCl, pH 7.4, 50mM $MgCl_2$, 500mM KCl) and 125 µl water. Aliquots of 15 µl were dispensed into tubes labeled 1-9 and chilled on ice. Exactly 4 units of Sau3A enzyme (10 units/µl BRL) were added to tube 1 and mixed well. Two-fold serial dilutions of the reaction mixtures from tube 1 sequentially through to tube 8 were performed to yield reaction mixtures containing two-fold decreasing concentrations of Sau3A enzyme. Tube 9 which contained no enzyme represented the undigested control reaction. The tubes were incubated at 37°C for 1 hour and stopped by returning the tubes to ice and adding EDTA (20mM). The extent of digestion in each tube was monitored by agarose gel electrophoresis, and the sample which yielded DNA fragments in the approximate range of 2-20 kb was scaled up 200 fold and the reaction repeated. The digested Rhodococcus 10554 DNA was separated by agarose gel

electrophoresis using low gelling temperature agarose (SeaPlaque, FMC). The region of the gel containing DNA fragments in the size range 4-8 kb was excised with a sterile scalpel and the DNA recovered by electro-elution. The DNA was precipitated by conventional methods, and the precipitate was resuspended in TE to a concentration of 0.025 mg/ml.

A single colony of S. lividans/pIJ702 was used to inoculate 50 ml of YEME which contained per liter: Difco yeast extract, 3g; Difco peptone, 5g; Oxoid malt extract, 3g; glucose, 10g; sucrose, 340g; $MgCl_2$ at 5mM; and glycine at 0.4% w/v; and was supplemented with the plasmid selective agent thiostrepton (CalBiochem), at 50 µg/ml. Other media described for Streptomyces could also be used. The culture was incubated at 30°C for 3 days. The cells were harvested by centrifugation at 10,000 rpm for 30 minutes using a Sorvall centrifuge and SS-34 rotor. The pellet was resuspended in 5ml TSE Buffer (25mM Tris, pH 8.0; 0.3M sucrose; 25mM EDTA) containing 10 mg lysozyme. The cells were incubated at 37°C for 30 minutes. A 3 ml volume of alkaline SDS solution (0.3M NaOH, 2% w/v sodium dodecyl sulfate) was added, and the mixture was incubated at room temperature for 10 minutes and at 70°C for 10 minutes. The lysed cell solution was cooled to room temperature and extracted with 1 volume of phenol: chloroform (1:1). The aqueous phase was removed to a clean tube and 0.3M sodium acetate, pH 4.8, and 1 volume of isopropanol added. The DNA was precipitated for 30 minutes at -20°C and recovered by centrifugation, using a Sorvall SS-34 rotor at 10,000 rpm for 10 minutes. The pellet was resuspended in 2 ml TE and the plasmid DNA purified by ultracentrifugation to equilibrium in a cesium chloride density gradi nt according to known techniques.

Plasmid pIJ702 prepared as above was resuspended in TE to a concentration of 1 mg/ml. About 10 µl (10 µg) of DNA was added to 5 µl 10x BglII buffer (500mM Tris HCl, pH 7.4; 50mM $MgCl_2$; 500mM KCl), 32 µl distilled water and 3 µl of BglII restriction enzyme (10 units/µl, BRL) and incubated for 2 hours at 37°C. The reaction was terminated by one extraction with phenol:chloroform (1:1) and one extraction with chloroform:isoamyl alcohol (24:1). The DNA was precipitated by the addition of one-half volume of 7.5M ammonium acetate and 2 volumes of cold ethanol. After incubation at -20°C for at least 2 hours, the DNA was collected in an Eppendorf microfuge by centrifugation for 10 minutes. The DNA pellet was washed once with 70% ethanol, dried and resuspended in 50 µl 10mM Tris HCl, pH 8.0. To dephosphorylate the D A, 1 µl of calf intestine alkaline phosphatase (2 U/µl, IBI) was added and the reaction incubated at 37°C for 30 minutes. The reaction was stopped by adding 150 µl stop buffer (10mM Tris HCl, pH 7.5; 1mM EDTA, pH 7.5; 200mM NaCl; 0.5% w/v sodium dodecyl sulfate) and then extracted once with phenol:chloroform (1:1) and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with ethanol, dried and resuspended in TE to a final concentration of 0.05 mg/ml and stored at -20°C.

About 10 µl (0.5 µg) of the dephosphorylated BglII digested pIJ702 DNA was added to about 20 µl (0.5 µg) of the Sau3A digested Rhodococcus DNA. A 20 µl aliquot of 5x ligase buffer (BRL), 0.5 µl ligase (NEB) and 50 µl water were added and the ligation reaction incubated at 14°C for 20 hours. The reaction was terminated by heating the mixture to 65°C for 10 minutes, and the sample was stored at -20°C.

A vegetative inoculum was prepared by growing S. lividans in 25 ml culture broth as described for the preparation of S. lividans/pIJ702, except the medium contained no thiostrepton. The cells were harvested in a benchtop centrifuge at 3,000 rpm for 10 minutes and washed twice with 10 ml of 10.3% w/v sucrose. The cell pellet was resuspended in 4ml lysozyme medium (L-medium, Thompson et al., 1982, J. Bacteriol., Vol. 151, pp. 668-677) and incubated at 30°C for 30 minutes. A 5 ml aliquot of protoplast medium (P-medium, Okanishi, et al., 1974, J. Gen. Microbiol., Vil. 80, pp. 389-400) was added and the solution mixed by pipetting up and down. Protoplasts were filtered through glass wool (which traps mycelia but allows protoplasts to pass through) harvested by centrifugation, and resuspended in 2 ml P-medium. Protoplasts were used fresh or stored frozen in P-medium at -70°C.

Up to 3 µl of ligated plasmid DNA in ligation buffer was mixed gently with 50 µl S. lividans protoplasts in a small sterile tube. To this mixture, 200 µl transformation buffer (T-buffer, Thompson, et al., 1982, J. Bacteriol., Vol. 151, pp. 668-667) was added and pipetted to mix. Immediately, the cells were spread gently onto R2YE medium (Thompson, et al., 1980, Nature (London), Vol. 286, pp. 525-527) and incubated overnight at 30°C. The regenerated protoplasts were overlaid with filter paper (Whatman 541, 9.0cm) containing 100 µg/ml thiostrepton and incubated at 30°C for 3 days. About 200-500 transformants per plate were obtained using these transformation conditions. Approximately 12,000 thiostrepton-resistant S. lividans transformants were obtained in this way. Over 70% of these transformants contained recombinant pIJ702 plasmids as estimated by miniprep DNA analysis. These

recombinant plasmids represented the genomic library of Rhodococcus 10554 DNA.

Screening for Cholesterol Oxidase Activity

As an example, showing the expression of one DNA sequence derived from a Nocardioform microorganism, recombinant S. lividans cells containing the genomic library of Rhodococcus 10554 DNA were screened for the expression of cholesterol oxidase activity. Cholesterol oxidase was determined using a modified method of Allain et al., 1974, Physical Chem., Vol. 20, pp. 470-475. Filter paper discs were soaked in an assay buffer containing 4-aminoantipyrine, phenol, horse radish peroxidase and triton-X100, and laid down on colonies growing on agar medium containing cholesterol substrate. Alternatively, these colonies could be assayed by halo formation on agar medium containing cholesterol, in which halo formation depended on the conversion of cholesterol to cholestenone by soluble cholesterol oxidase. Recombinant S. lividans colonies which produced cholesterol oxidase were purified, and characterized.

Details of the screening of the genomic library for cholesterol oxidase activity were as follows: Filter papers used to apply thiostrepton drug to R2YE transformation plates for the selection of S. lividans transformants, were lifted and placed onto screening plates [medium containing per liter: agar, 15g; glycerol, 20g; yeast extract, 1g; $MgSO_4$, 0.66g; buffer salts, 66 ml (per liter: Na_2HPO_4, 28g; NaH_2PO_4, 30g; K_2HPO_4, 20g; $(NH_4)_2SO_4$, 127.5 g) and trace salts, 2 ml (per liter: concentrated HCl, 250 ml; $CaCl_2$, 3.57g; $ZnSO_4$, 20g; $CuCl_2$, 0.85g; $NaMoO_4$, 4.8g; $MnCl_2$, 2.0g; $FeCl_3$, 5.4g; boric acid, 0.3g; $CoCl_2$, 2.4g)] with cholesterol (1.6mM, Sigma) and

thiostrepton (50 µg/ml). These replica plates were incubated at 30°C for 3 days. The replica filters were removed and replaced with sterile filter paper discs soaked in cholesterol oxidase assay reagent (100mM citric acid buffer, pH 6.0; 0.2mM 4-aminoantipyrine (Aldrich); horse radish peroxidase I, 600 units per liter; 6.4mM cholesterol; 10mM phenol and 4% w/v Triton x 100). The plates were incubated at 30°C, and the colonies were screened over a 4 hour period for the development of a red zone due to cholesterol oxidase activity which diffused onto the white filter paper. After incubation at 30°C for 4 hours, the assay reagent filter papers were removed from the screening plates, and the plates were re-incubated at 30°C overnight to allow for the formation of clear halos associated with cholesterol oxidase degradation of cholesterol. From the approximately 8,000 recombinant S. lividans colonies screened, two showed cholesterol oxidase activity. The plasmid DNA from one of these isolates was called pSL81 and was analyzed further.

Plasmid DNA from the S. lividans/pSL81 recombinant was isolated and transformed back into S. lividans using the same procedure described for isolating and transforming pIJ702. All the thiostrepton-resistant transformants obtained contained the same plasmid pIJ702 with approximately 6 kb insert DNA, and showed the cholesterol oxidase activity diagnostic of the original recombinant. Curing of plasmid pSL81 from S. lividans cells by growth in the absence of drug selective pressure resulted in the simultaneous loss of thiostrepton resistance and cholesterol oxidase activity. Southern hybridization demonstrated that the insert DNA was derived from the Rhodococcus 10554 genome. Referring to Fig. 1, the

recombinant plasmid designated pSL81 includes cloning vector pIJ702 12 and insert DNA 14 from Rhodococcus 10554, and has restriction endonuclease sites as indicated.

Plasmid pSL81, prepared as above, was used to probe chromosomal digests from the cholesterol oxidase producing Nocardioforms Rhodococcus NCIB 10554, R. erythropolis NCIB 9158, Nocardia erythropolis ATCC 17895 and Nocardia erythropolis ATCC 4277. The strains all showed cross-hybridizing DNA bands suggesting that the cholesterol oxidase coding region in these Nocardioforms is the same. Plasmid pSL81 showed no specific cross hybridization with DNA isolated from a cholesterol oxidase producing Streptomyces, namely Streptomyces violascene NRRL B-2700.

DNA Sequencing

In order to sequence the cholesterol oxidase gene, subclones of pSL81 were constructed and a cholesterol oxidase coding region was localized to a 2.8 kb BglII fragment in the subclone pSL81Δ15 (Fig. 3). This fragment was then introduced into E. coli on plasmid pBR322, and a series of deletion derivatives were constructed using available restriction sites. Sequencing was performed on denatured double stranded plasmid DNA using the dideoxy chain termination method of Sanger et al., PNAS USA, Vol. 74, pp. 5463-67. Specifically, a Sequenase Version 2.0 kit (United States Biochemical Corp.) was used according to the manufacturer's specifications. Deoxyguanidine triphosphate or Deoxy-7-deazoguanidine triphosphate was used to terminate chain polymerization reactions depending on the degree of G+C content of a particular region of DNA being sequenced. The primer extended oligonucleotides were electrophoresed on denaturing 6%

polyacrylamide and the DNA sequences of adjacent DNA fragments were pieced together to construct a complete DNA sequence of the cholesterol oxidase gene. The sequence is shown in Fig. 2.

Subcloning of the Rhodococcus cholesterol oxidase gene in pSL81Δ15 can result in an increase in expression of the cholesterol oxidase protein. Recombinant S. lividans cells were grown on RZYE agar containing 50µg/ml thiostrepton. After 2-3 days incubation at 30°C, the mycelia and spores were scraped from the surface of the agar and used to inoculate 50 ml YEME + 12.5 µg/ml thiostrepton seed cultures. These cultures were grown at 30°C with vigorous shaking and after 48 h, 2 l fermenters containing YEME + 12.5 µg/ml thiostrepton were inoculated with the seed. The fermentations were controlled at 28°C and pH 7.0 with aeration at 0.5 l/min and agitation at 1000 rpm. Cholesterol oxidase activity peaked after 48 h and in some such cultures expression was substantially greater in the S. lividans/pSL81Δ15 fermentations than in the S. lividans/pSL81 fermentations.

Protein characterization

Recombinant cholesterol oxidase was isolated from 3 day old cultures of S. lividans/pSL81 cells grown in YEME (25 ml) containing thiostrepton (50 µg/ml). Non-recombinant cholesterol oxidase was isolated from 48 hour Rhodococcus 10554 cultures grown in liquid production medium which was the same medium as that used for the screening plates not solidified with agar. Induction experiments were performed using the same growth conditions in the presence of sterol as an inducer. Intracellular and extracellular fractions were recovered by centrifugation and assayed for cholesterol oxidase activity using a cholesterol oxidase specific diagnostic assay. The specificity of the recombinant

cholesterol oxidase from S. lividans and the purified cholesterol oxidase from Rhodococcus 10554 were compared using various sterol substrates. Both enzymes showed similar profiles with the order of activity being cholesterol, pregnenolone, dihydroxycholesterol, stigmasterol and ergosterol.

Recombinant cholesterol oxidase from S. lividans/pSL81 was functional in standard diagnostic assays for the determination of cholesterol. An aliquot of cell-free supernatant from a 3 day old culture of YEME grown recombinant cells was mixed with the cholesterol oxidase assay reagent (1.4 ml of 0.33 mg/ml 4-aminophenazone in 0.1M phosphate buffer, pH 7.0, 0.05% Triton X-100 (w/v); 1.4 ml of 1 mg/ml phenol in phosphate-Triton X buffer; 0.1 ml of 4mM cholesterol; and 0.01 ml of 10 mg/ml peroxidase) and incubated at 30°C. Hydrogen peroxide formed during the oxidation of cholesterol and chelated with the aminoantipyrine in the assay mixture was determined by measuring the absorption of the red complex at 505nm versus a reagent blank containing water. The rate of cholesterol degradation by the recombinant enzyme was also measured directly by following the increase in absorbance at 240 nm owing to Δ^4-cholestenone production. To 2.9 ml of sodium phosphate buffer (0.1M, pH 7.0) in a cuvette, 100 µl of 3% Triton X-100 (w/v) and 50 µl of a 6mM cholesterol solution were added. Diluted enzyme at approximately 0.2 U/ml in buffer was added and the absorbance at 240nm monitored using a Beckman spectrophotometer. Cholesterol oxidase activity was calculated as:

$$\text{units/mg} = \frac{\text{OD/min} \times \text{RV}}{12.3 \times \text{DF} \times \text{SV} \times \text{PC (mg/ml)}},$$

where

RV = reaction volume
DF = dilution factor
SV = sample volume
PC = protein concentration

Further characterization of the recombinant cholesterol oxidase from S. lividans/pSL81 demonstrated that the enzyme could also be used to determine cholesterol in sera using the peroxidase coupled reaction.

Recombinant cholesterol oxidase from S. lividans/pSL81 and purified Rhodococcus 10554 cholesterol oxidase were analyzed by polyacrylamide gel electrophoresis using a 10% polyacrylamide gel and the buffer system of Laemelli, 1970, Nature, Vol. 227, pp. 680 et seq. Both enzymes were determined to be approximately 55 kD in size when compared with Biorad molecular weight standards.

Peptide sequencing

Recombinant cholesterol oxidase from S. lividans/pSL81Δ15 and non-recombinant cholesterol oxidase from R. rhodococcus were isolated as single bands from a polyacrylamide gel, and the amino-terminal peptide sequences were determined using a protein sequenator. Two peptide sequences were recovered from the recombinant protein band:

NH_2-Gly-Gly-Pro-Val-Ser-Thr-Leu-Thr-Pro-Pro-Pro-Ala-Phe-,

and

NH_2-Gly-Pro-Val-Ser-Thr-Leu-Thr-Pro-Pro-Pro-Ala-Phe-.

These peptide sequences are identical to those deduced directly from the DNA sequence between positions 280 and 320, and between positions 283 and 320, respectively. The elimination of a glycine residue at the N-terminus of one recombinant sequence is probably owing to a

difference in post-translational processing by the host cell.

Amino terminal sequencing of cholesterol oxidase from R. rhodococcus identified the sequence NH_2-Thr-Pro-Pro-Pro-Ala-Phe-Pro-Glu-Gly-Ile-Ala-Leu-Tyr-Gln-Gln-, which corresponds to the peptide sequence deduced from the DNA sequence between positions 300 and 346. Therefore, the enzyme found in the S. lividans/pSL81Δ15 culture medium is either 6 or 7 amino acids longer that the membrane bound enzyme found in R. rhodococcus.

Processing of gram-positive secreted proteins often involves signal sequence cleavage followed by hydrolysis at a secondary processing site. In the case of native cholesterol oxidase this secondary site is at the threonine. Apparently such a secondary cleavage does not occur when the protein is synthesized and secreted extracellularly by S. lividans.

Other Embodiments

Other embodiments are within the following claims. For example, other species of any genus of the Nocardioforms including Rhodococcus NCIB 10554, R. erythropolis NCIB 9158, Nocardia erythropolis ATCC 17895 and Nocardia erythropolis ATCC 4277 could be used to prepare the genomic library; or the libraries could be screened for other useful metabolic activities such as the assimilation of alkanes and aromatic hydrocarbons; the degradation of lignin, detergents, and pesticides; the production of enzymes useful in xenobiotic transformation; the biosynthesis of antibiotics, amino acids and biosurfactants; and the production of other steroid modifying enzymes such as cholesterol esterase.

As other Streptomyces and also Bacillus species

share the advantages of S. lividans for the expression of heterologous genes, they could also serve as suitable hosts for the expression of DNA sequences derived from Nocardioform microorganisms. Other bacteria belonging to the high G+C subdivision of the gram-positive bacteria including any of the Nocardioforms, Mycobacterium, Corynebacterium, and Athrobacter would be suitable hosts for cloning DNA sequences derived from Nocardioform microorganisms.

S. lividans/pSL81 can undergo rearrangement in one or more regions outside the cholesterol oxidase coding region and can retain cholesterol oxidase transforming activity. One such S. lividans/pSL81 rearranged plasmid, has at least 3.6 kb of the 6 kb Rhodococcus DNA unchanged as determined by restriction mapping. In this rearranged S. lividans/pSL81 plasmid a DNA insertion of approximately 2.5 kb has occurred in the region to the right of the PvuII site. Cholesterol oxidase was recoverable from cultures of S. lividans transformed with this rearranged plasmid, in lower quantities than from S. lividans/pSL81 cultures.

In still other embodiments expression of cholesterol oxidase may be increased by further manipulating the pSL81Δ15 clone, as will be apparent to one skilled in the art. For example, a higher copy number vector could be used, such as, for example, pIJ303, described in Hopwood et al., 1985, Genetic Manipulation of Streptomyces: A Laboratory Manual. Gene expression could also be enhanced by replacing the cholesterol oxidase promoter with a stronger exogenous promoters, such as ErmE promoter, described in Bibb et al., 1985, Gene, Vol. 38, pp. 215-226; aph promoter, described in Hopwood et al., 1985; or a tac promoter,

described in Amman et al., 1984, Gene, Vol. 25, pp. 167 et seq.

Deposit

Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, deposit of plasmid pSL81 has been made with the American Type Culture Collection (ATCC) of Rockville, MD, USA, where the deposit was given Accession Number _____.

Applicants' assignee, Genzyme Corporation, represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. § 122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited plasmid, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicants' assignee acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

Claims

1. A cloning vector comprising a DNA sequence derived from a Nocardioform microorganism, said DNA sequence encoding a protein, and said cloning vector being capable of expressing said DNA sequence in a host microorganism.

2. The cloning vector of claim 1 wherein said Nocardioform microorganism is Rhodococcus sp. NCIB 10554.

3. The cloning vector of claim 1 wherein said host microorganism is a gram-positive microorganism.

4. The cloning vector of claim 1 wherein said host microorganism is a species of Streptomyces.

5. The cloning vector of claim 1 wherein said host microorganism is S. lividans.

6. The cloning vector of claim 1 wherein said DNA sequence encodes cholesterol oxidase.

7. The plasmid pSL81.

8. A microorganism transformed with the cloning vector of claim 1 or 2.

9. A gram-positive microorganism transformed with the cloning vector of claim 3.

10. A cell of a species of Streptomyces transformed with the cloning vector of claim 4.

11. A cell of the species S. lividans transformed with the cloning vector of claim 5.

12. A method for expressing a DNA sequence derived from a Nocardioform microorganism comprising

providing a cloning vector containing said DNA sequence,

transforming a host microbial cell with said cloning vector to obtain a recombinant host cell, and

culturing said recombinant host cell under conditions permitting expression of said DNA sequence.

13. A method for producing cholesterol oxidase comprising

providing a cloning vector containing a DNA sequence derived from a Nocardioform microorganism, said DNA sequence encoding cholesterol oxidase,

transforming a host cell with said cloning vector to obtain a recombinant host cell,

culturing said recombinant host cell under conditions permitting expression of said DNA sequence, and

recovering the cholesterol oxidase.

14. The method of claim 13 wherein said Nocardioform organism is Rhodococcus sp. NCIB 10554.

15. The method of claim 13 wherein said host cell is a gram-positive microorganism.

16. The method of claim 13 wherein said host cell is a Streptomyces sp.

17. The method of claim 13 wherein said host cell is S. lividans.

18. The method of claim 13 wherein said cloning vector is plasmid pSL81.

19. Recombinant cholesterol oxidase encoded by DNA derived from a Nocardioform microorganism.

20. Recombinant cholesterol oxidase encoded by DNA derived from Rhodococcus sp. NCIB 10554.

21. Cholesterol oxidase of claim 19 or 20, essentially free of lipids found in said Nocardioform microorganism.

22. Cholesterol oxidase of claim 19 or 20 produced in a transformed heterologous host microorganism.

23. Cholesterol oxidase of claim 19 or 20 produced in a transformed host gram-positive microorganism.

24. Cholesterol oxidase of claim 19 or 20 produced in a transformed species of Streptomyces.

25. Cholesterol oxidase of claim 19 or 20 produced in transformed S. lividans.

26. Cholesterol oxidase of claim 19 or 20 produced substantially in extracellular form.

27. Cholesterol oxidase of claim 19 or 20 expressed without the addition of inducer.

28. A cell transformed with a DNA sequence derived from a Nocardioform microorganism, said DNA sequence encoding cholesterol oxidase.

29. A method for the determination of cholesterol in a sample of fluid from a human comprising

contacting said sample with recombinant cholesterol oxidase encoded by DNA derived from a Nocardioform microorganism and

determining the extent of oxidation of cholesterol.

30. The method of claim 30 wherein determining the extent of oxidation of cholesterol comprises

measuring the consumption of oxygen with an oxygen electrode.

31. The method of claim 30 wherein determining the extent of oxidation of cholesterol comprises

measuring the production of hydrogen peroxide.

32. The method of claim 30 wherein determining the extent of oxidation of cholesterol comprises

spectrophotometrically determining the conversion of cholesterol to Δ^4-cholestenone.

33. The cloning vector of claim 1, wherein said DNA sequence encodes an amino acid sequence comprising the sequence:

Thr Pro Pro Pro Ala Phe Pro Glu Gly Ile Ala Leu Tyr Gln Gln.

34. The cloning vector of claim 33, wherein said DNA sequence encodes an amino acid sequence comprising the sequence:

Gly Pro Val Ser Thr Leu Thr Pro Pro Pro Ala Phe Pro Glu Gly Ile Ala Leu Tyr Gln Gln.

35. The cloning vector of claim 34, wherein said DNA sequence encodes an amino acid sequence comprising the sequence:

Gly Gly Pro Val Ser Thr Leu Thr Pro Pro Pro Ala Phe Pro Glu Gly Ile Ala Leu Tyr Gln Gln.

36. The cloning vector of claim 35, wherein said DNA sequence encodes an amino acid sequence comprising the sequence:

Met Thr Ala Gln Asp Glu Lys Phe Arg Leu Ser Arg Arg Gly Phe Met Ala Ala Gly Ala Gly Ala Val Ala Ala Thr Ala Phe Ala Gly Trp Thr Pro Ala Tyr Ala Val Pro Ala Gly Ser Ser Gly Ser Ala Gly Gly Pro Val Ser Thr Leu Thr Pro Pro Pro Ala Phe Pro Glu Gly Ile Ala Leu Tyr Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met Leu Asp.

37. The cloning vector of claim 1, wherein said DNA sequence comprises the sequence:

ACA CCG CCG CCC GCC TTC CCC GAA GGC ATC GCG CTG TAC CAG CAG

38. The cloning vector of claim 37, wherein said DNA sequence comprises the sequence:

GGT CCT GTC TCC ACC CTC ACA CCG CCG CCC GCC TTC CCC GAA GGC ATC GCG CTG TAC CAG CAG

39. The cloning vector of claim 38, wherein said DNA sequence comprises the sequence:

GGT GGT CCT GTC TCC ACC CTC ACA CCG CCG CCC GCC TTC CCC GAA GGC ATC GCG CTG TAC CAG CAG

40. The cloning vector of claim 39, wherein said DNA sequence comprises the sequence:

CGAAG ATG ACG GCA CAA GAC GAA AAG TTC CGA CTG TCC CGA CGA GGT TTC ATG GCC GCT GGA GCC GGC GCC GTG GCA GCG ACC GCA TTC GCC GGC TGG ACG CCG GCC TAC GCC GTC CCC GCC GGC TCC TCC GGC TCC GCG GGT GGT CCT GTC TCC ACC CTC ACA CCG CCG CCC GCC TTC CCC GAA GGC ATC GCG CTG TAC CAG CAG GCA TAT CAG AAC TGG TCC AAA GAG ATC ATG CTC GAC

41. The cloning vector of claim 40, wherein said DNA sequence comprises the sequence:

GGGACTCCTG ATCTCAGCTT CCGTACTGGA GCGCGAAGCT CCTGCCCTGG CTGACGTAGT TCTCACTCTT GTCTGATACC AACCTGTCTG ATACCCACCT GTTAGAACTC ACCGTAGTTC TCGAACCCGA TGGAGTAGCC CGAAG ATG ACG GCA CAA GAC GAA AAG TTC CGA CTG TCC CGA CGA GGT TTC ATG GCC GCT GGA GCC GGC GCC GTG GCA GCG ACC GCA TTC GCC GGC TGG ACG CCG GCC TAC GCC GTC CCC GCC GGC TCC TCC GGC TCC GCG GGT GGT CCT GTC TCC ACC CTC ACA CCG CCG CCC GCC TTC CCC GAA GGC ATC GCG CTG TAC CAG CAG GCA TAT CAG AAC TGG TCC AAA GAG ATC ATG CTC GAC

42. The cloning vector of claim 1 wherein said Nocardioform microorganism is R. erythropolis NCIB 9158.

43. The cloning vector of claim 1 wherein said Nocardioform microorganism is Nocardia erythropolis ATCC 17895.

44. The cloning vector of claim 1 wherein said Nocardioform microorganism is Nocardia erythropolis ATCC 4277.

45. The method of claim 13 wherein said cloning vector is plasmid pIJ303.

46. The vector of claim 1, further comprising an ErmE promoter.

47. The vector of claim 1, further comprising an aph promoter.

48. The vector of claim 1, further comprising a tac promoter.

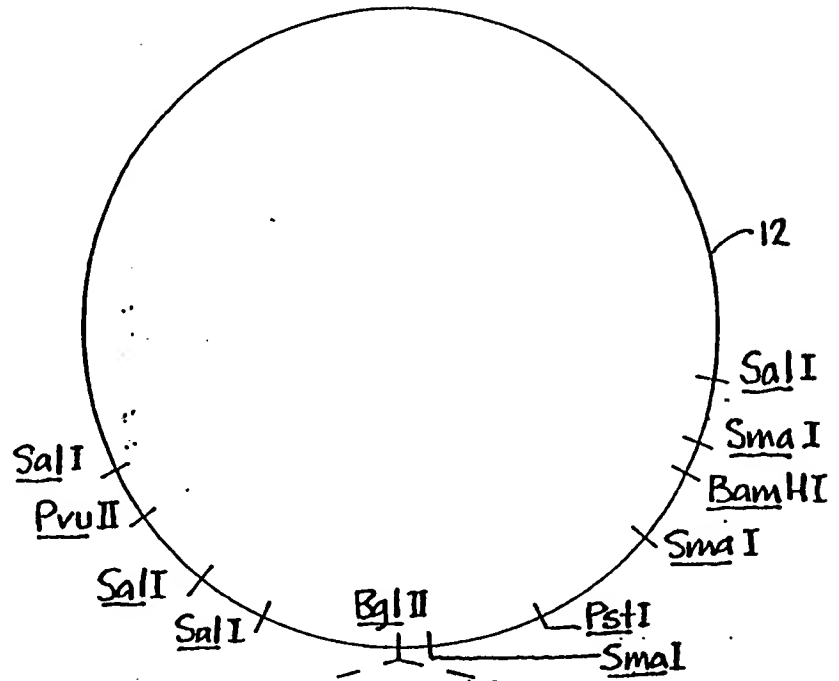
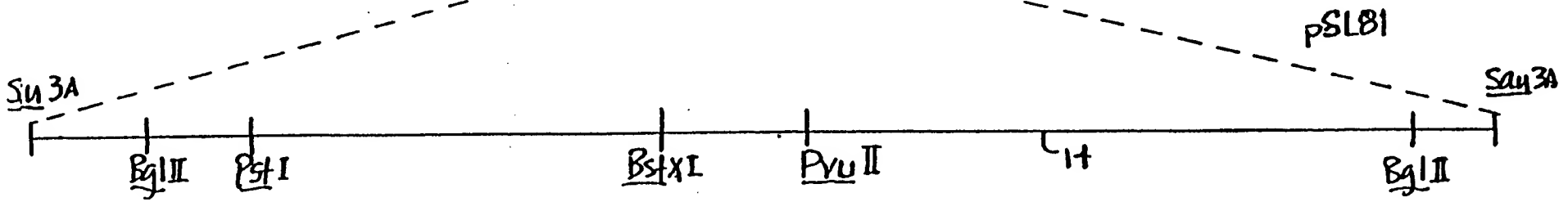

12
SalI
SmaI
BamHI
SmaI
PstI
SmaI
BglII
SalI
SalI
PvuII
SalI

FIG. 1


pSL81
Sau3A
Sau3A
BglII
PstI
BstXI
PvuII
1t
BglII

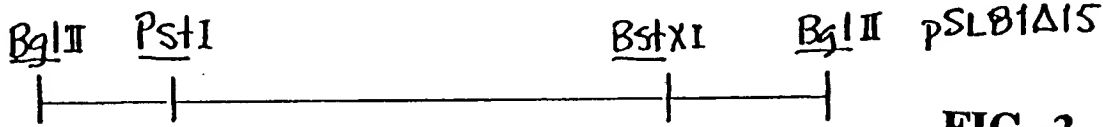

BglII
PstI
BstXI
BglII
pSL81Δ15

FIG. 3

Translation of CO2 over region 146-2029;.

```
      10        20        30        40        50        60        70
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GGGACTCCTG ATCTCAGCTT CCGTACTGGA GCGCGAAGCT CCTGCCCTGG CTGACGTAGT TCTCACTCTT
CCCTGAGGAC TAGAGTCGAA GGCATGACCT CGCGCTTCGA GGACGGGACC GACTGCATCA AGAGTGAGAA

      80        90       100       110       120       130       140
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GTCTGATACC AACCTGTCTG ATACCCACCT GTTAGAACTC ACCGTAGTTC TCGAACCCGA TGGAGTAGCC
CAGACTATGG TTGGACAGAC TATGGGTGGA CAATCTTGAG TGGCATCAAG AGCTTGGGCT ACCTCATCGG

      150       160       170       180       190       200
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CGAAG ATG ACG GCA CAA GAC GAA AAG TTC CGA CTG TCC CGA CGA GGT TTC ATG GCC GCT GGA
GCTTC TAC TGC CGT GTT CTG CTT TTC AAG GCT GAC AGG GCT GCT CCA AAG TAC CGG CGA CCT
     Met Thr Ala Gln Asp Glu Lys Phe Arg Leu Ser Arg Arg Gly Phe Met Ala Ala Gly→
     ____a__a__a__a__a__a__AUTOTRANSLATION OF CO2__a__a__a__a__a__a__→

      210       220       230       240       250       260
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GCC GGC GCC GTG GCA GCG ACC GCA TTC GCC GGC TGG ACG CCG GCC TAC GCC GTC CCC GCC
CGG CCG CGG CAC CGT CGC TGG CGT AAG CGG CCG ACC TGC GGC CGG ATG CGG CAG GGG CGG
Ala Gly Ala Val Ala Ala Thr Ala Phe Ala Gly Trp Thr Pro Ala Tyr Ala Val Pro Ala→
____a__a__a__a__a__a__AUTOTRANSLATION OF CO2__a__a__a__a__a__a__→

      270       280       290       300       310       320
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GGC TCC TCC GGC TCC GCG GGT GGT CCT GTC TCC ACC CTC ACA CCG CCG CCC GCC TTC CCC
CCG AGG AGG CCG AGG CGC CCA CCA GGA CAG AGG TGG GAG TGT GGC GGC GGG CGG AAG GGG
Gly Ser Ser Gly Ser Ala Gly Gly Pro Val Ser Thr Leu Thr Pro Pro Pro Ala Phe Pro→
____a__a__a__a__a__a__AUTOTRANSLATION OF CO2__a__a__a__a__a__a__→

      330       340       350       360       370       380
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GAA GGC ATC GCG CTG TAC CAG CAG GCA TAT CAG AAC TGG TCC AAA GAG ATC ATG CTC GAC
CTT CCG TAG CGC GAC ATG GTC GTC CGT ATA GTC TTG ACC AGG TTT CTC TAG TAC GAG CTG
Glu Gly Ile Ala Leu Tyr Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met Leu Asp→
____a__a__a__a__a__a__AUTOTRANSLATION OF CO2__a__a__a__a__a__a__→
```

FIG. 2.1

```
      390         400         410         420         430         440
       *           *           *           *           *           *
GCG ATC TGG ACC TGT TCA CCC AAG ACG CCC GAA GAC GTA GTC GCC TCG CGA ACT GGG CCA
CGC TAG ACC TGG ACA AGT GGG TTC TGC GGG CTT CTG CAT CAG CGG AGC GCT TGA CCC GGT
Ala Ile Trp Thr Cys Ser Pro Lys Thr Pro Glu Asp Val Val Ala Ser Arg Thr Gly Pro→
 ___n___n___n___n___n___n___AUTOTRANSLATION OF CO2___n___n___n___n___n___n___n___→

      450         460         470         480         490         500
       *           *           *           *           *           *
TCC AAC GGC TAC ACC ATT CGT CCC CGC GGG CCA TGC GTG GAC GCC GCT GAC CAT CGT CAA
AGG TTG CCG ATG TGG TAA GCA GGG GCG CCC GGT ACG CAC CTG CGG CGA CTG GTA GCA GTT
Ser Asn Gly Tyr Thr Ile Arg Pro Arg Gly Pro Cys Val Asp Ala Ala Asp His Arg Gln→
 ___n___n___n___n___n___n___AUTOTRANSLATION OF CO2___n___n___n___n___n___n___n___→

      510         520         530         540         550         560
       *           *           *           *           *           *
CGG TGC GCC GGT CGA CAA GGT CAT CCT CGC CGA CAC CAC GGT CCA CCT CAC CGG CGT CTC
GCC ACG CGG CCA GCT GTT CCA GTA GGA GCG GCT GTG GTG CCA GGT GGA GTG GCC GCA GAG
Arg Cys Ala Gly Arg Gln Gly His Pro Arg Arg His His Gly Pro Pro His Arg Arg Leu→
 ___n___n___n___n___n___n___AUTOTRANSLATION OF CO2___n___n___n___n___n___n___n___→

      570         580         590         600         610         620
       *           *           *           *           *           *
CGT CAA CGC CGG TGG CAG CCC GGC CAC CGT CAC CGC AGG ACC CGG CGC GAC CCT CGA CGC
GCA GTT GCG GCC ACC GTC GGG CCG GTG GCA GTG GCG TCC TGG GCC GCG CTG GGA GCT GCG
Arg Gln Arg Arg Trp Gln Pro Gly His Arg His Arg Arg Thr Arg Arg Asp Pro Arg Arg→
 ___n___n___n___n___n___n___AUTOTRANSLATION OF CO2___n___n___n___n___n___n___n___→

      630         640         650         660         670         680
       *           *           *           *           *           *
CAT CAC CAC CGC ACT GCA GGC ACA GGG CCT CGG GTT CGC GAA CTG CCG GCG CCC GGT GTG
GTA GTG GTG GCG TGA CGT CCG TGT CCC GGA GCC CAA GCG CTT GAC GGC CGC GGG CCA CAC
His His His Arg Thr Ala Gly Thr Gly Pro Arg Val Arg Glu Leu Pro Ala Pro Gly Val→
 ___n___n___n___n___n___n___AUTOTRANSLATION OF CO2___n___n___n___n___n___n___n___→

      690         700         710         720         730         740
       *           *           *           *           *           *
TTG ACC ATC GCC GGC TGC CTC GCC GTC GAC GCT CAC GGT GCA GCG CTC CCC GCC GAA GGC
AAC TGG TAG CGG CCG ACG GAG CGG CAG CTG CGA GTG CCA CGT CGC GAG GGG CGG CTT CCG
Leu Thr Ile Ala Gly Cys Leu Ala Val Asp Ala His Gly Ala Ala Leu Pro Ala Glu Gly→
 ___n___n___n___n___n___n___AUTOTRANSLATION OF CO2___n___n___n___n___n___n___n___→

      750         760         770         780         790         800
       *           *           *           *           *           *
GAA GCA CAC GTT CCC GGA CAG ACT TTC GGC TCA CTC TCC AAC CTC GTC ACC TCC CTG ACC
CTT CGT GTG CAA GGG CCT GTC TGA AAG CCG AGT GAG AGG TTG GAG CAG TGG AGG GAC TGG
Glu Ala His Val Pro Gly Gln Thr Phe Gly Ser Leu Ser Asn Leu Val Thr Ser Leu Thr→
 ___n___n___n___n___n___n___AUTOTRANSLATION OF CO2___n___n___n___n___n___n___n___→
```

FIG. 2.2

```
      810         820         830         840         850         860
  *     *     *     *     *     *     *           *     *     *     *
GCA GTG GTC TGG AAC GGC AGT GAG TAC GCG CTC GAG ACG TAC GCG CGT AGC GAT GCA GCG
CGT CAC CAG ACC TTG CCG TCA CTC ATG CGC GAG CTC TGC ATG CGC GCA TCG CTA CGT CGC
Ala Val Val Trp Asn Gly Ser Glu Tyr Ala Leu Glu Thr Tyr Ala Arg Ser Asp Ala Ala→
___A__A__A__A__A__A___AUTOTRANSLATION OF CO2_A__A__A__A__A__A__A__→

      870         880         890         900         910         920
  *     *     *     *     *     *     *     *     *     *     *     *
ATC AAG CCG CTG CTG ACT CAC CTC GGA CGC ACC TTC CTC ACC TCC GTG ACC TTG CAG GCC
TAG TTC GGC GAC GAC TGA GTG GAG CCT GCG TGG AAG GAG TGG AGG CAC TGG AAC GTC CGG
Ile Lys Pro Leu Leu Thr His Leu Gly Arg Thr Phe Leu Thr Ser Val Thr Leu Gln Ala→
___A__A__A__A__A__A___AUTOTRANSLATION OF CO2_A__A__A__A__A__A__A__→

      930         940         950         960         970         980
  *     *     *     *     *     *     *     *     *     *     *     *
GCT CCC AAC TAC CGC ATG CGC TGC GTC AGC CAC ACC GAC ATC GGT TGG CAG GAA CTC TTC
CGA GGG TTG ATG GCG TAC GCG ACG CAG TCG GTG TGG CTG TAG CCA ACC GTC CTT GAG AAG
Ala Pro Asn Tyr Arg Met Arg Cys Val Ser His Thr Asp Ile Gly Trp Gln Glu Leu Phe→
___A__A__A__A__A__A___AUTOTRANSLATION OF CO2_A__A__A__A__A__A__A__→

      990         1000        1010        1020        1030        1040
  *     *     *     *     *     *     *     *     *     *     *     *
GGC GCC CGC GGA GCG TCC GGA CGC ACC TTC GAG AAG TTC GTC CGC GAA AAC GGT CGC GCA
CCG CGG GCG CCT CGC AGG CCT GCG TGG AAG CTC TTC AAG CAG GCG CTT TTG CCA GCG CGT
Gly Ala Arg Gly Ala Ser Gly Arg Thr Phe Glu Lys Phe Val Arg Glu Asn Gly Arg Ala→
___A__A__A__A__A__A___AUTOTRANSLATION OF CO2_A__A__A__A__A__A__A__→

      1050        1060        1070        1080        1090        1100
  *     *     *     *     *     *     *     *     *     *     *     *
GAA GCA ATC TGG TAC CCC TTC ACC GAA CGC CCG TGG ATG AAG GTG TGG TCA CTT GCC CCC
CTT CGT TAG ACC ATG GGG AAG TGG CTT GCG GGC ACC TAC TTC CAC ACC AGT GAA CGG GGG
Glu Ala Ile Trp Tyr Pro Phe Thr Glu Arg Pro Trp Met Lys Val Trp Ser Leu Ala Pro→
___A__A__A__A__A__A___AUTOTRANSLATION OF CO2_A__A__A__A__A__A__A__→

      1110        1120        1130        1140        1150        1160
  *     *     *     *     *     *     *     *     *     *     *     *
ACC AAG CGG CCG TTC TCG CGT GAG GTG ACC GGG CCC TAC AAC TAC ATC TTC TCC GAC AAC
TGG TTC GCC GGC AAG AGC GCA CTC CAC TGG CCC GGG ATG TTG ATG TAG AAG AGG CTG TTG
Thr Lys Arg Pro Phe Ser Arg Glu Val Thr Gly Pro Tyr Asn Tyr Ile Phe Ser Asp Asn→
___A__A__A__A__A__A___AUTOTRANSLATION OF CO2_A__A__A__A__A__A__A__→

      1170        1180        1190        1200        1210        1220
  *     *     *     *     *     *     *     *     *     *     *     *
CTC CCG GAG CCG GTC ACC GAC ATG ATC GGG CAG ATC AAC GCC GGA AAC CCG GGC ATC GCA
GAG GGC CTC GGC CAG TGG CTG TAC TAG CCC GTC TAG TTG CGG CCT TTG GGC CCG TAG CGT
Leu Pro Glu Pro Val Thr Asp Met Ile Gly Gln Ile Asn Ala Gly Asn Pro Gly Ile Ala→
___A__A__A__A__A__A___AUTOTRANSLATION OF CO2_A__A__A__A__A__A__A__→
```

FIG. 2.3

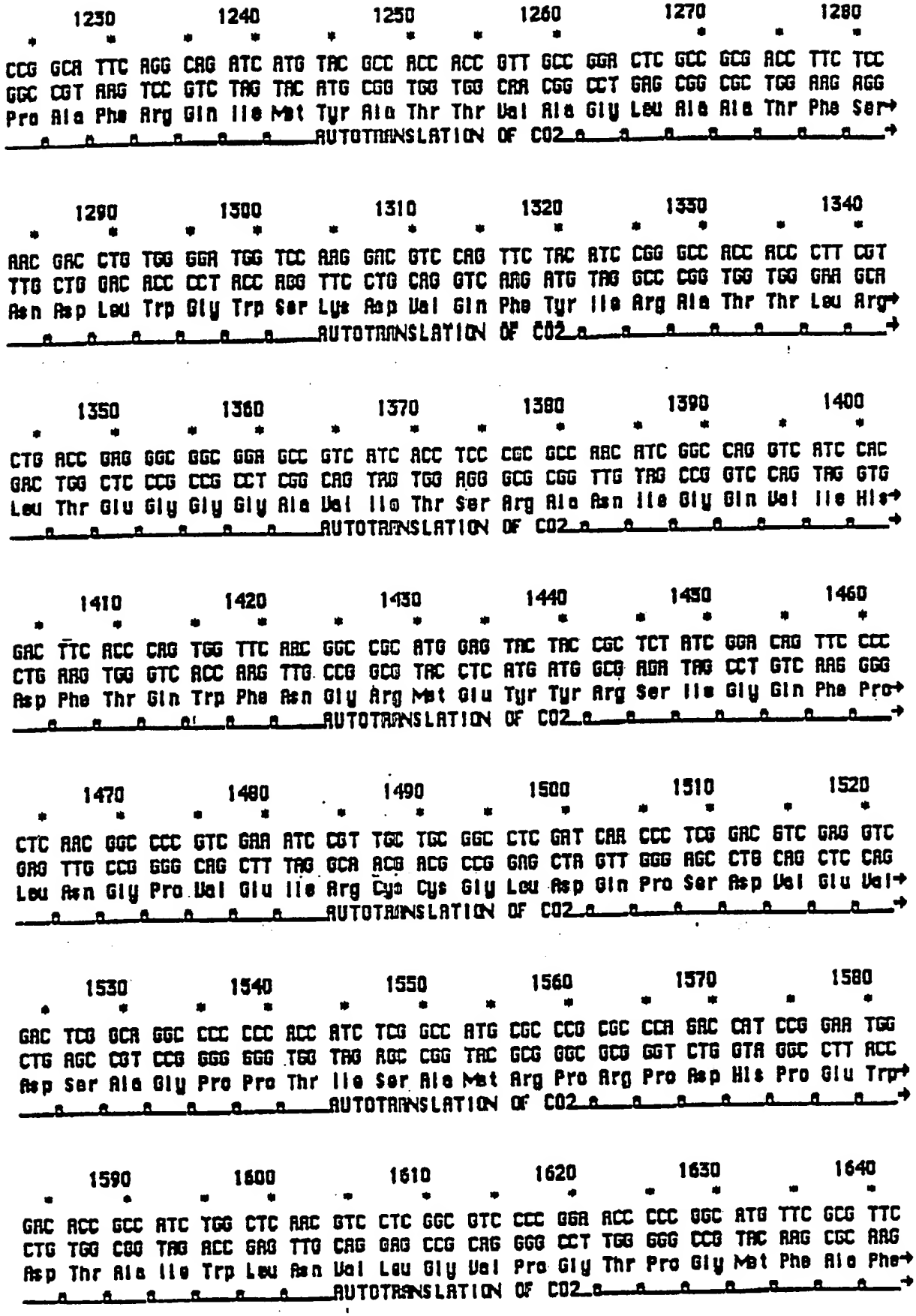

1230 1240 1250 1260 1270 1280
CCG GCA TTC AGG CAG ATC ATG TAC GCC ACC ACC GTT GCC GGA CTC GCC GCG ACC TTC TCC
GGC CGT AAG TCC GTC TAG TAC ATG CGG TGG TGG CAA CGG CCT GAG CGG CGC TGG AAG AGG
Pro Ala Phe Arg Gln Ile Met Tyr Ala Thr Thr Val Ala Gly Leu Ala Ala Thr Phe Ser
AUTOTRANSLATION OF CO2
1290 1300 1310 1320 1330 1340
AAC GAC CTG TGG GGA TGG TCC AAG GAC GTC CAG TTC TAC ATC CGG GCC ACC ACC CTT CGT
TTG CTG GAC ACC CCT ACC AGG TTC CTG CAG GTC AAG ATG TAG GCC CGG TGG TGG GAA GCA
Asn Asp Leu Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr Ile Arg Ala Thr Thr Leu Arg
AUTOTRANSLATION OF CO2
1350 1360 1370 1380 1390 1400
CTG ACC GAG GGC GGC GGA GCC GTC ATC ACC TCC CGC GCC AAC ATC GGC CAG GTC ATC CAC
GAC TGG CTC CCG CCG CCT CGG CAG TAG TGG AGG GCG CGG TTG TAG CCG GTC CAG TAG GTG
Leu Thr Glu Gly Gly Gly Ala Val Ile Thr Ser Arg Ala Asn Ile Gly Gln Val Ile His
AUTOTRANSLATION OF CO2
1410 1420 1430 1440 1450 1460
GAC TTC ACC CAG TGG TTC AAC GGC CGC ATG GAG TAC TAC CGC TCT ATC GGA CAG TTC CCC
CTG AAG TGG GTC ACC AAG TTG CCG GCG TAC CTC ATG ATG GCG AGA TAG CCT GTC AAG GGG
Asp Phe Thr Gln Trp Phe Asn Gly Arg Met Glu Tyr Tyr Arg Ser Ile Gly Gln Phe Pro
AUTOTRANSLATION OF CO2
1470 1480 1490 1500 1510 1520
CTC AAC GGC CCC GTC GAA ATC CGT TGC TGC GGC CTC GAT CAA CCC TCG GAC GTC GAG GTC
GAG TTG CCG GGG CAG CTT TAG GCA ACG ACG CCG GAG CTA GTT GGG AGC CTG CAG CTC CAG
Leu Asn Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp Gln Pro Ser Asp Val Glu Val
AUTOTRANSLATION OF CO2
1530 1540 1550 1560 1570 1580
GAC TCG GCA GGC CCC CCC ACC ATC TCG GCC ATG CGC CCG CGC CCA GAC CAT CCG GAA TGG
CTG AGC CGT CCG GGG GGG TGG TAG AGC CGG TAC GCG GGC GCG GGT CTG GTA GGC CTT ACC
Asp Ser Ala Gly Pro Pro Thr Ile Ser Ala Met Arg Pro Arg Pro Asp His Pro Glu Trp
AUTOTRANSLATION OF CO2
1590 1600 1610 1620 1630 1640
GAC ACC GCC ATC TGG CTC AAC GTC CTC GGC GTC CCC GGA ACC CCC GGC ATG TTC GCG TTC
CTG TGG CGG TAG ACC GAG TTG CAG GAG CCG CAG GGG CCT TGG GGG CCG TAC AAG CGC AAG
Asp Thr Ala Ile Trp Leu Asn Val Leu Gly Val Pro Gly Thr Pro Gly Met Phe Ala Phe
AUTOTRANSLATION OF CO2

FIG. 2.4

```
     1650        1660        1670        1680        1690        1700
      *     *     *     *     *     *     *     *     *     *     *     *
TAC CGC GAA ATG GAA CAG TGG ATG CCT AAT CAC TAC AAC AAC AAC GAC GCC ACC TTC CGC
ATG GCG CTT TAC CTT GTC ACC TAC GGA TTA GTG ATG TTG TTG TTG CTG CGG TGG AAG GCG
Tyr Arg Glu Met Glu Gln Trp Met Pro Asn His Tyr Asn Asn Asn Asp Ala Thr Phe Arg→
___a__a__a__a__a__a___AUTOTRANSLATION OF CO2_a__a__a__a__a__a__a__→

     1710        1720        1730        1740        1750        1760
      *     *     *     *     *     *     *     *     *     *     *     *
CCA GAA TGG TCC AAG GGC TGG CGT TCG GCC CCG ACA AGC CGT ACA CCG ACG CCC CGA TCA
GGT CTT ACC AGG TTC CCG ACC GCA AGC CGG GGC TGT TCG GCA TGT GGC TGC GGG GCT AGT
Pro Glu Trp Ser Lys Gly Trp Arg Ser Ala Pro Thr Ser Arg Thr Pro Thr Pro Arg Ser→
___a__a__a__a__a__a___AUTOTRANSLATION OF CO2_a__a__a__a__a__a__a__→

     1770        1780        1790        1800        1810        1820
      *     *     *     *     *     *     *     *     *     *     *     *
TCA CCC AGG GCC TCC CGC AGA CCT ACC GCG ACG GCG TCC CAT CGA GCG ACA ACT GGG ACA
AGT GGG TCC CGG AGG GCG TCT GGA TGG CGC TGC CGC AGG GTA GCT CGC TGT TGA CCC TGT
Ser Pro Arg Ala Ser Arg Arg Pro Thr Ala Thr Ala Ser His Arg Ala Thr Thr Gly Thr→
___a__a__a__a__a__a___AUTOTRANSLATION OF CO2_a__a__a__a__a__a__a__→

     1830        1840        1850        1860        1870        1880
      *     *     *     *     *     *     *     *     *     *     *     *
CCG CCA ACG CCG CAT ACA ACG CGT TGG ATC CGC ACA AGG TCT TCA GCA ACA CCT TCC TGG
GGC GGT TGC GGC GTA TGT TGC GCA AGC TAG GCG TGT TCC AGA AGT CGT TGT GGA AGG ACC
Pro Pro Thr Pro His Thr Thr Arg Trp Ile Arg Thr Arg Ser Ser Ala Thr Pro Ser Trp→
___a__a__a__a__a__a___AUTOTRANSLATION OF CO2_a__a__a__a__a__a__a__→

     1890        1900        1910        1920        1930        1940
      *     *     *     *     *     *     *     *     *     *     *     *
ACC AGT TGC TGC CCT GAG GTC CAA GCA CCA GGC ACG CAC TCT GCC AAC TCG ACG GTC GTC
TGG TCA ACG ACG GGA CTC CAG GTT CGT GGT CCG TGC GTG AGA CGG TTG AGC TGC CAG CAG
Thr Ser Cys Cys Pro Glu Val Gln Ala Pro Gly Thr His Ser Ala Asn Ser Thr Val Val→
___a__a__a__a__a__a___AUTOTRANSLATION OF CO2_a__a__a__a__a__a__a__→

     1950        1960        1970        1980        1990        2000
      *     *     *     *     *     *     *     *     *     *     *     *
GCC ACT CCC CTA CTT TCG GGA GTG CGG CCG CCG TCG TGC GTT GCA GTG AGC GGG ATG AAG
CGG TGA GGG GAT GAA AGC CCT CAC GCC GGC GGC AGC ACG CAA CGT CAC TCG CCC TAC TTC
Ala Thr Pro Leu Leu Ser Gly Val Arg Pro Pro Ser Cys Val Ala Val Ser Gly Met Lys→
___a__a__a__a__a__a___AUTOTRANSLATION OF CO2_a__a__a__a__a__a__a__→

     2010        2020        2030        2040        2050        2060        2070
      *     *     *     *     *     *     *     *     *     *     *     *     *
GAG TCC CCC CGC ATC CCG ACC AGG TAA C TGACTCAATC GCGATCAGGA TGGATCGCCT CGTGCATACT
CTC AGG GGG GCG TAG GGC TGG TCC ATT G ACTGAGTTAG CGCTAGTCCT ACCTAGCGGA GCACGTATGA
Glu Ser Pro Arg Ile Pro Thr Arg End→
___a__AUTOTRANSLATION OF CO2___a___→
```

FIG. 2.5

```
     2080       2090       2100       2110       2120       2130       2140
      *  *       *  *       *  *       *  *       *  *       *          *  *
TTCCACGGCC CTGGCATGAG TTGAGAGCTC GGACCGAGCC GACCGAATCG TGGCGCTGTC GCTGCGCGCC
AAGGTGCCGG GACCGTACTC AACTCTCGAG CCTGGCTCGG CTGGCTTAGC ACCGCGACAG CGACGCGCGG

     2150       2160       2170       2180       2190       2200       2210
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CCCGGCTCGG ACGACGACGA GTCCTCTCCG GGGTTCGACG CGCCACCAGC GGGATGCTCG GAGGAGCCGG
GGGCCGAGCC TGCTGCTGCT CAGGAGAGGC CCCAAGCTGC GCGGTGGTCG CCCTACGAGC CTCCTCGGCC

     2220       2230       2240       2250       2260       2270       2280
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGCGCTCTTC CCTCTCCGCG CGGGCGCGCA ACAACGACTC GGACTTCGGG GTATTTCGCA TCGGAGCATT
ACGCGAGAAG GGAGAGGCGC GCCCGCGCGT TGTTGCTGAG CCTGAAGCCC CATAAAGCGT AGCCTCGTAA

     2290       2300       2310       2320       2330
      *  *       *  *       *  *       *  *       *  *       *
GTCCCAGATT CATCTCCCAT GACTCACATG GAGCGCTCTC ACCCCCGGTG ACCAGGCC
CAGGGTCTAA GTAGAGGGTA CTGAGTGTAC CTCGCGAGAG TGGGGGCCAC TGGTCCGG
```

FIG. 2.6

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/60; C12Q 1/44; C12Q 1/26; C12N 15/09; C12N 9/02; C12N 1/21; U.S.Cl.: 435/11, 19, 25, 28, 172.3, 189, 252.3, 320

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/ 11, 19, 25, 28, 172.3, 189, 252.3, 320 935/ 9, 22, 60, 72

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstract Database (CA): 1967-1990; Biosis Database 1969-1990; Keywords: choesterol oxidase, Rhodococcus, Streptomyces, vector, plasmid, gene

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	JOURNAL OF BACTERIOLOGY; Volume 170, issued 1988, February, (M.E. VOGT SINGER), "Construction of an Escherichia coli-Rhodococcus shuttle vector and plasmid transformation in Rhodococcus spp.", See pages 638-645	1 and 12 2-11, 13-28 and 33-48
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Volume 52, issued 1986, December, (Y. MUROOKA), "Cloning and expression of a Streptomyces cholesterol oxidase gene in Streptomyces lividans with plasmid pIJ702", See pages 1382-1385	1-48
Y	U.S., A., 4,409,326, (I.E. MODROVICH), 11 October 1983, See columns 5-6	29-32

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6 FEBRUARY 1990	21 FEB 1990

International Searching Authority	Signature of Authorized Officer
ISA/US	RICHARD C. PEET